Immunocytochemical localization of vasopressin-binding sites in the rat kidney

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ABSTRACT

The rat kidney and brain are major target organs for vasopressin (VP). A procedure was developed for immunocytochemical staining of VP and its binding sites in the kidney. This procedure involved preincubation of kidney sections with the ligand, followed by immunocytochemical detection of VP.

The staining in renal tubules from Wistar rats was obtained in the thick ascending limb of the loop of Henle. There was no staining under any circumstances in proximal tubules. In the kidney of the Brattleboro rat homozygous for hypothyroid diabetes insipidus (DI) which congenitally lacks VP but responds to the peptide, exactly the same staining pattern was observed after preincubation with VP, but the maximal staining was less intense. The VP binding to the DI rat kidney, after 2 weeks treatment with VP (using Accurel implants), reached levels seen in the Wistar kidney after in-vitro preincubation with high doses of VP.

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INTRODUCTION

Vasopressin (VP) is a neuropeptide produced in the brain and released from the neurohypophysis into the blood where it acts as an antidiuretic hormone. In addition, it is transported into the brain, released from synapses and affects central regulatory processes including thermoregulation, cardiovascular homeostasis, memory and learning (Buijs, 1982). In general, hormones and neurotransmitters first interact with specific binding sites to express their functional effects. Binding sites can be localized either by using antibodies against the receptor component or by detecting the bound ligand using two possible approaches: autoradiography or immunocytochemistry.

An immunocytochemical localization of binding sites may have various advantages over autoradiography (e.g. Dorsa, Majumdar, Petracca et al. 1983). Thus the former can be applied to a large number of related peptides for which no suitable radioactive labelled ligand is available, it has a higher resolution at the light microscopical level, and it may also be examined electron microscopically.

Since antibodies to VP receptors are not available at present, the approach of Sternberger, Petralli, Joseph & Mills (1978) to immunocytochemical localization of luteinizing hormone-releasing hormone-binding sites has been applied to localize renal VP-binding sites, a well-established site of action of VP.

The presence of endogenous peptide is likely to be a problem in the immunocytochemical detection of peptide-binding sites and an additional methodological advantage in the localization of VP-binding sites is, therefore, the use of the VP-deficient mutant Brattleboro (DI) rat with hypothalamic diabetes insipidus (Valtin & Schroeder, 1964) in which exogenous VP exerts its normal effects on kidney and brain.

A lower VP binding was observed in the kidney of the DI rat when compared with the kidney of the Wistar rat. The binding in the DI kidney was thus studied after 2 weeks of VP treatment, using implanted Accurel tube containing different doses of VP (Boer, Kruisbrink & Van Pelt Heerschap, 1983). The long-term release of VP increased renal binding of VP in the DI rat to the levels observed in the normal Wistar rat.
MATERIALS AND METHODS

Animals

Twenty-five male Wistar white rats and twelve DI Brattleboro rats, homozygous for diabetes insipidus, weighing 200–300 g, were obtained from CPB/TNO (Zeist, The Netherlands). All animals had free access to standard chow and tap water and were kept under a light regime of 12 h light: 12 h darkness (lights on at 07.00 h). Diabetes insipidus was diagnosed on the basis of daily water consumption of individually caged rats (Valtin & Schroeder, 1964; Swaab, Boer & Nolten, 1973).

DI' rats were implanted subcutaneously in the neck with Accurel tubing loaded with an aqueous solution of arginine vasopressin (AVP; grade VIII, 430 units/mg) under light ether anaesthesia. Accurel polypropylene tubing was supplied by Mr D. Heitman, ENKA Research Laboratories, Obernburg, F.R.G. Twelve rats were divided into three groups; group A was implanted with tubing filled with 20 µg AVP, group B with tubing containing 200 µg AVP and group C with water-filled tubing. Water consumption was measured in each rat 4 days before and 2 weeks after implantation. The rats were perfused and the kidneys stained as described below.

Tissue fixation, ligand preincubation and immunocytochemical staining

Rats were perfused intracardiacally under sodium pentobarbitone anaesthesia (0.1 mg/100 g body weight, i.p.), first with a rapid wash of saline followed by 500 ml freshly prepared fixative. Several fixatives were used: (1) Bouin–Holland sublimate; (2) 2% paraformaldehyde, 1–25% glutaraldehyde, 0–025% CaCl₂ and 0–5% picric acid in 0–1 M-cacodylate buffer, pH 7–4; (3) 2–5% glutaraldehyde and 1–0% paraformaldehyde in 0–1 M-cacodylate buffer, pH 7–4. After removal, the kidneys were decapsulated, cut into halves and kept in the fixative at 4°C. Twenty-four hours later the fixative was renewed, and the kidneys were stored in it until staining, 1–3 weeks later. No loss of VP-binding capacity was observed after 4 months of storage in fixative. Before staining the kidneys were cut transversely into 40 µm thick sections on a vibratome (Oxford Instruments), and free-floating sections were washed for 24 h at 4°C, with 0–05 M-Tris-buffered saline (TBS), pH 7–6.

Kidney sections cut on the vibratome were washed in TBS for 90 min (6 × 15 min on a rotating table) and then incubated with lysine (0–1 mol/l) for 1 h to inactivate the remaining reactive glutaraldehyde groups. This was followed by washing in TBS (3 × 10 min) and incubation with the appropriate peptide, dissolved in TBS, for 30 min. All incubations were performed at room temperature unless stated otherwise. The sections were washed for 10 min in TBS and 10 min in cacodylate buffer (0–1 mol/l) and subsequently post-fixed with 2% glutaraldehyde in cacodylate (0–1 mol/l) for 15 min. This short post-fixation step was introduced to prevent washing out of VP from its binding sites during immunocytochemical staining and washing. Excess glutaraldehyde was washed away (10 min in cacodylate, 0–1 mol/l) after which the sections were rinsed for 15 min with lysine (0–1 mol/l) in Tris buffer. This was followed by rinsing with incubation buffer (3 × 15 min). The incubation buffer consisted of 0–05 M-TBS containing 0–5% Triton X-100. The sections were incubated with the first antibody, diluted in incubation buffer, for 1 h at room temperature, followed by 26–72 h at 4°C. The peroxidase-anti-peroxidase (PAP) technique was used for staining, using AVP antibody at a dilution of 1:1000 as first antibody, unless stated otherwise. After this incubation the sections were washed with incubation buffer (3 × 20 min). The incubation with the second antibody (goat-anti-rabbit immunoglobulin G serum, Betsy) diluted 1:50 in incubation buffer, lasted for 1–5 h. After rinsing with incubation buffer (3 × 15 min), the sections were incubated for 1–5 h with the PAP complex, diluted 1:1000, and washed (2 × 15 min) with incubation buffer and (1 × 15 min) with 0–05 M-Tris–HCl buffer, pH 7–6. Peroxidase activity was detected by exposing the sections to 0–5 mg 3–3′-diaminobenzidine (DAB)/ml and H₂O₂ (0–01%) in 0–05 M-Tris–HCl buffer, pH 7–6, for 10 min at room temperature. The DAB reaction was stopped by extensive washing in buffer (2 × 10 min) and the sections were mounted on glass slides and allowed to dry overnight at room temperature. The next day they were dehydrated in graded ethanol and embedded in entellan.

The specificities of the VP binding and the immunocytochemical procedures were assessed as follows. (1) The first antibody was replaced by pre-immune serum (Ducky 1:1000). (2) A first antibody was used which had been preadsorbed with the homologous peptide. VP antibody (WI) was preadsorbed with VP coupled covalently to cyanogen–bromide-activated agarose beads (Swaab & Pool 1975; Pool, Buijs, Swaab et al. 1983). The preadsorbed antibody was used at a dilution of 1:500 and the quantitative ratio between AVP and the agarose-coated beads was 200 µg AVP/ml beads. (3) Renal sections were incubated with different peptides (oxytocin; melanocyte-stimulating hormone (MSH); arginine vasotocin (AVT)) and subsequently stained with their homologous antibodies (anti-oxytocin; anti-MSH; anti-AVT). (4) Sections were co-incubated with VP and an analogue of VP. The analogue used, des-gly-amide-AVP (dgAVP), has low antiidiuretic activity (Rigter &
entire study. Each point represents the mean of five different sections (n = 5) taken from two kidneys of two rats, which were selected at random. Any statement in the Results regarding a difference in staining intensity between two experimental groups is made only when there was no overlap in the scores of the two groups.

**Peptides and antisera**

Arginine vasopressin (grade VIII) was obtained from Sigma, St Louis, MO, U.S.A.; dgAVP (B587) from Bioproducts Peptide Department, Belgium; oxytocin (grade X) from Sigma; α-MSH (WB 1138 K1) and AVT (TH 223 K11) from Organon, Oss, The Netherlands. Vasopressin antibody (W1) was kindly donated by Dr. Tj. B. van Wimersma Greidanus, Rudolph Magnus Institute, Utrecht, The Netherlands; vasopressin antibody (126, 19.3.73), antioxytocin (O-1-V, 4.4.75), anti-AVT (8-82, 9.2.82), anti-MSH (4394, 9.4.75) and pre-immune serum (Ducky 26.6.80) were all raised in our laboratory.

**RESULTS**

Before applying the immunocytochemical procedure for staining VP-binding sites in the kidney, a tissue fixative was sought to yield the optimal binding capacity for VP. The best results were obtained with a combination of 2-5% glutaraldehyde and 1-0% paraformaldehyde, in cacodylate (0-1 mol/l) buffer, pH 7.4. This fixative was used for further studies.

The same ligand preincubation and staining procedures were performed on paraffin wax-embedded sections (6-8 μm), vibratome sections (40 μm) and cryostat sections (50 μm). The optimal staining was observed on vibratome sections. The time-dependence for saturation of VP-binding sites was checked and the optimal time found to be 30 min. No differences in staining intensity or localization of VP-binding sites were found between the two different VP antibodies (126; W1) employed for Wistar and DI rats under all preincubation conditions. The staining intensities outlined in the Plate were scored according to the system described in Materials and Methods.

**Staining of endogenous VP in Wistar and DI rat kidney**

A very low level of staining was observed in sections of Wistar and DI rat kidney not preincubated with VP and stained with a VP antibody. This occasional staining was a little greater in the Wistar kidney compared with the DI kidney (Text-fig. 2 and Plate, figs 3, 4) and observed only in a small percentage of medullary segments of collecting ducts.

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Staining after preincubation with VP

The staining was enhanced when kidney sections were preincubated with VP and stained with VP antibody. Text-figure 2 shows the staining intensities in relation to VP concentration in the Wistar and the DI kidney stained with a VP antibody at a dilution of 1:1000. The staining was seen most prominently in the epithelia of the distal convoluted tubules and collecting ducts, both medullary and cortical portions (Plate, figs 1, 2). No staining was observed in control sections treated with anti-VP serum, which had been adsorbed with AVP. From the concentration response-curves (Text-fig. 2) it appears that the antiserum, at 1:1000 dilution, on sections of Wistar kidney (Text-fig. 2a), yielded maximal staining intensity at a VP concentration of 0.06 μg/ml (60 nmol/l). In contrast the maximum staining intensity of the DI kidney sections (Text-fig. 2b) was reached with this antiserum at a VP concentration of 0.6 μg/ml (600 nmol/l).

The staining of the epithelia of the collecting ducts and the distal convoluted tubules was more pronounced in the apical than in the basal portions of the cells (Plate). Only after preincubation with high VP concentrations (0.6–6.0 μg/ml; 600–6000 nmol/l) was a moderate and marked staining observed in the distal convoluted tubules.

Under no circumstances was any staining observed in the epithelia of the proximal convoluted tubules in either Wistar or DI kidneys.

Staining in the DI rat after VP replacement from Accurel VP implants

Water consumption dropped immediately after VP implantation in both groups of rats. In group A, implanted with a tube containing 20 μg VP, releasing 0.2 μg VP/24 h, it remained low for 5 days and began to increase on day 6. In group B, implanted with 200 μg VP, releasing 2 μg VP/24 h, water consumption stayed low for 8 days and started to rise on day 9, indicating that the higher dose in the Accurel tubing resulted in a longer period of release of AVP. The control group C was implanted with water-filled tubing. Kidney sections from these three groups were stained immunocytochemically for AVP both with and without VP preincubation. In the control group, there was an occasional staining without preincubation, and a marked staining of collecting ducts and distal convoluted tubules after preincubation with 0.6 μg VP/ml. Kidney sections of group A (treated with the low dose of VP) showed weak staining of cortical and medullary collecting ducts (similar to the DI kidney preincubated with the lowest dose; Text-fig. 2b) and occasionally of distal convoluted tubules without preincubation. In sections from the same kidney which had been preincubated with VP, the staining intensity was marked in both collecting ducts (similar to the DI kidney preincubated with the high dose; Text-fig. 2b) and distal convoluted tubules. The kidney sections from group B (treated with the high dose of VP) showed areas of marked staining of medullary and cortical collecting ducts (similar to maximum staining in the DI kidney after preincubation with high dose; Text-fig. 2b), and moderate staining of distal convoluted tubules, without preincubation with VP. Sections from the same group which had been preincubated with VP before staining showed areas of very intense staining of collecting ducts in cortex and medulla, as well as in the distal convoluted tubules. In this group, the staining of medullary collecting ducts was similar to the maximum staining of Wistar kidney (Text-fig. 2a) and the cortical collecting ducts and
distal convoluted tubules were even more intensely stained than the same ducts in the Wistar kidney preincubated with a high dose of VP (Text-fig. 2a).

Specificity control experiments
Replacement of the first antibody by pre-immune serum (Ducky 1 : 1000) always resulted in an absence of staining in both Wistar and DI kidneys either preincubated with VP or not.

Preadsorption of VP antibody with VP (1 : 500) with or without preincubation of the sections with VP resulted in complete absence of staining in both Wistar and DI kidneys (Plate, figs 5,6).

Incubation of kidney sections with different peptides resulted in similar weak staining in Wistar and DI kidneys. Only a small percentage of medullary portions of collecting ducts was stained with oxytocin and MSH, and the weak staining intensities were not enhanced by preincubation with increasing peptide concentrations (0–0.6 μg/ml). The staining with AVT was intermediate between the staining levels observed with oxytocin and AVP.

Staining with anti-VP (1 : 1000) after co-incubation of the sections with VP and dgAVP is shown in Text-fig. 3. After preincubation with dgAVP alone, weak staining in the DI and moderate staining of medullary collecting ducts in the Wistar kidney was observed (Text-fig. 3a). When increasing amounts of AVP were added, together with a constant amount of dgAVP (0.6 μg/ml), 0.6 μg AVP/ml was needed to obtain marked staining of collecting ducts, and only at an AVP concentration of 0.6 μg/ml was a marked staining of distal tubules observed (Text-fig. 3b). Addition of dgAVP with a constant amount of AVP (0.6 μg/ml) caused a sharp decline in staining intensities, in medullary and cortical collecting ducts and distal tubules, at 0.6 μg dgAVP/ml.

The blocking effect of dgAVP was identical in Wistar and DI kidneys.

DISCUSSION

The present immunocytochemical localization of VP-binding sites largely agrees with the preliminary data reported by Sternberger (1979), Ravid & Castel (1981) and Ravid, Castel & Borut (1982). Our preliminary studies lacked sensitivity and reproducibility, problems that were overcome in the present study by improved fixation, incubation and staining procedures. The localization agrees with the extensive literature on the physiological site of action of antidiuretic hormone. Vasopressin alters the concentration of urine by changing the water permeability of the distal convoluted tubules and the collecting ducts (see review by Handler & Orloff, 1981).

**TEXT-Figure 3.** Staining intensity of vasopressin (VP)-binding sites in Wistar rat kidney sections cut on a vibratome and preincubated with arginine vasopressin (AVP) and des-gly-amide-VP (dgAVP) in concentrations from 0 to 60 μg/ml and stained with anti-VP diluted 1 : 1000. (a) AVP (0) + dgAVP (0–60 μg/ml); (b) dgAVP (constant 0.6 μg/ml) + AVP (0–60 μg/ml); (c) AVP (constant 0.6 μg/ml) + dgAVP (0–60 μg/ml). The different types of renal tubules are: medullary collecting ducts (MCD), cortical collecting ducts (CCD) and distal convoluted tubules (DC). Staining intensities: — no staining, + occasional staining, + weak staining, ++ moderate staining, +++ marked staining and ++++ very intense staining.
Darmady, Durant, Matthews & Stranack (1960) located $^{131}$I-labelled pitressin in rat kidney by autoradiography, and found marked activity in the distal convoluted tubules and the collecting ducts. Localization of radioactivity at a cellular level could not be determined by this technique however. Fahrenholz, Thierauch & Crause (1980) and Crause & Fahrenholz (1982), using affinity labelling, found binding sites for VP in the inner medulla of bovine kidney. Dorsa et al. (1983) and Van Leeuwen & Wolters (1983) found intense labelling in the renal medulla and moderate labelling in the renal cortex of the rat, but again the resolution of the technique did not permit conclusions as to the cellular localization of VP-binding sites.

In the present paper, renal VP binding sites have been identified which are significantly enhanced immunocytochemically after preincubation with VP. The distal nephron comprises three structurally distinct segments: the distal convoluted tubule, the connecting tubule and the cortical collecting duct. Distal convoluted tubules are present only in cortex, while thick ascending limbs of Henle’s loop (TAL) are present in both cortex and outer medulla. Our studies correlate this morphology with differential VP-binding capacities, and show that there is a gradual decline in VP binding along the different segments of the nephron. The cortical parts had a lower binding capacity for VP compared with the medullary and papillary parts. Imbert-Teboul, Chabardes, Montegut et al. (1978) suggested that the in-vivo concentrations of VP necessary to induce stimulation of the membrane-bound adenylate cyclase activity are higher for TAL than for collecting ducts. Such also applies to the immunocytochemical staining after VP pretreatment in vitro; the staining in distal convoluted tubules was enhanced with the medium concentrations of added VP but only the use of high concentrations of VP during the preincubation caused a significant enhancement of staining in the TAL. The total absence of staining in the renal proximal tubules concurs with the physiological data for the site of VP action.

At first glance the more intense staining observed on the luminal side of the tubular epithelium does not seem to be in agreement with the reported site of action of VP on the basolateral membrane (Jard, 1980). Since hormone binding is reversible, there may be loss of hormone bound to basolateral receptors during the two 10-min washing steps before post-fixation. Even if such a loss takes place, the hormone bound to this site was clearly stained in our sections. Moreover, the washing time employed was comparable to that used in autoradiographic binding assays of VP in rat kidney and brain (Van Leeuwen & Wolters, 1983). In addition, the localization agrees with the site of cellular changes, due to VP, seen on the electron microscope after VP treatment. Brown & Orci (1983) reported that VP stimulated the formation of coated pits in luminal membranes of collecting ducts in the kidney of DJ rats. Coated pits are known to be involved in receptor-mediated endocytosis and to function as transport organelles that carry selected receptor-bound proteins into the cells (Goldstein, Anderson & Brown, 1979). The binding to luminal membranes may represent an "internalization" process of a receptor–peptide complex in the kidney (Brown, Grosso & De Sousa, 1980) and toad urinary bladder (Muller, Kachadorian & Discala, 1980). Vasopressin also stimulates formation of intramembrane particle (IMP) aggregates, which are transferred from the cytoplasm to luminal membranes (Wade, Guckian & Koeppen, 1984). However, the direct relationship between these changes in luminal membranes of collecting ducts and VP has still to be clarified.

We observed an increase in staining intensities either with or without preincubation with VP in vitro in DI rats implanted with Accurel tubing containing VP. The effect of VP on stimulating adenylate cyclase activity in plasma membranes of renal medulla from DI rats has been studied by Doussa, Hui & Barnes (1975) and Rajerison, Butlen & Jard (1977). Both groups showed that medullo-papillary adenylate cyclase sensitivity to VP was reduced in the DI rat, while chronic VP treatment restored normal sensitivity. These findings may suggest that the presence of binding sites and the responsiveness of adenylate cyclase are both regulated by the level of circulating peptide. There are additional parallels between our results using VP substitution and those obtained by others. Harrington & Valtin (1968) reported physiological changes in DI rat kidney after chronic VP treatment. Kriz & Bankir (1982) showed morphological changes in the DI rat kidney, caused by long-term treatment with VP. In this paper immunocytochemical staining of VP on its binding sites is enhanced in the DI rat kidney after treatment with VP.

The absence of staining in both Wistar and DI kidneys, after replacement of the first antibody by preimmune serum, excludes the possibility of non-specificity of the method. Negative staining in Wistar and DI kidneys, after replacement of the first antibody by anti-VP preadsorbed with VP, excludes staining due to antibodies other than those that are able to bind to AVP. The low basic level of staining which is present in the kidney sections without adding VP could result from the presence of endogenous VP in the Wistar kidney. The occasional staining in the DI kidney might be due to oxytocin, which circulates at higher concentrations in this mutant rat (Dogterom, Van Wimersma Greidanus & Swaab, 1977) and is mildly antidiuretic (Sawyer & Valtin, 1967). Arginine vasotocin is an antidiuretic principle in non-mammalian vertebrates (Manning, Grzonka &

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Sawyer, (1981). Gilmore, Wesley, Huffman & Nemeh (1982) showed that AVT had a direct effect on collecting ducts of dog kidney and increased tubular epithelial permeability to water. The intermediate staining after preincubation with AVT agrees with the antidiuretic activity of this peptide.

α-Melanocyte-stimulating hormone (α-MSH) has been reported to have natriuretic activity and may regulate sodium balance (Thody, 1980). The exact mechanisms and the sites of action for such effects are not yet known.

An important point for the specificity control experiments is the exclusion of possible neurophysin staining in the kidney (Ciarochi, Haluszczak & Robinson, 1976). Since DI rats do not produce VP-linked neurophysin (Brownstein & Gainer, 1977) it is certain that after VP preincubation staining of AVP-binding sites was not due to neurophysin.

The displacement of AVP from its renal receptor by the analogue dgAVP, as reported here, is interesting for various reasons. dgAVP is generally claimed to have no antidiuretic activity in rats and man, and to affect memory and learning processes directly by a central action without affecting water and electrolyte metabolism, blood pressure and pulse rate (De Wied, Greven, Lande & Witter, 1972; Laczi, Van Rees, Wagner et al. 1983). Recently, however, this peptide was shown to have some antidiuretic activity, e.g. by Rigter & Crabbe (1982) in the DI rat. In the present experiments it seems that dgAVP binds to the same site as VP and the binding affinity towards dgAVP is quite high. Therefore the reported actions of dgAVP might be due, at least in part, to peripheral effects. If the binding of dgAVP is also strong in vivo it might act antagonistically towards VP. If this is so, extreme precautions should be taken in the clinical use of this analogue, e.g. for the treatment of patients with memory disturbances. This holds true especially for aged patients, in whom VP binding in the kidney already seems to be reduced (R. Ravid, E. Fliers & D. F. Swaab, unpublished results).

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**DESCRIPTION OF PLATE**

Staining of vasopressin (VP)-binding sites in kidney sections cut on a vibratome of (Figs 1 and 3) Brattleboro rats homozygous for hypothalamic diabetes insipidus (DI) and (Figs 2 and 4) Wistar rats.

**FIGURES 1 and 2.** Sections preincubated with 0.6 μg arginine vasopressin (AVP)/ml (600 nmol/l) and stained with anti-VP (1:1000). Both figures show longitudinal sections of collecting ducts (×250). Insets: Higher magnification (×400) showing distal convoluted tubules in cortex.

**FIGURES 3 and 4.** Sections not preincubated with AVP and stained with anti-VP (1:1000) showing collecting ducts and thick ascending limbs of Henle's loop (×250).

**FIGURES 5 and 6.** Sections preincubated with AVP (0.6 μg/ml) and stained with anti-VP (1:500, preadsorbed with AVP beads and showing longitudinal sections of collecting ducts (×250).